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Saturation Transfer Difference Nuclear Magnetic Resonance Spectroscopy As a Method for Screening Proteins for Anesthetic Binding

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ABSTRACT

The effects of anesthetics on cellular function may result from direct interactions between anesthetic molecules and proteins. These interactions have a low affinity and are difficult to characterize. To identify proteins that bind anesthetics, we used nuclear magnetic resonance saturation transfer difference (STD) spectroscopy. The method is based on the nuclear Overhauser effect between bound anesthetic protons and all protein protons. To establish STD as a method for testing anesthetic binding to proteins, we conducted measurements on a series of protein/anesthetic solutions studied before by other methods. STD was able to identify that volatile anesthetics bind to bovine serum albumin, oleic acid reduces halothane binding to bovine serum albumin, and halothane binds to apomyoglobin but not lysozyme. Using STD, we found that halothane binding to calmodulin is Ca^{2+}-dependent, which demonstrates anesthetic specificity for a protein conformation. Thus, STD is a powerful tool for investigating anesthetic-protein interactions because of its abilities to detect weak binding, to screen a single protein for binding of multiple anesthetics simultaneously, and to detect a change in anesthetic binding caused by conformational changes or competition with other ligands.

The cellular targets of anesthetics and the anesthetic mechanisms of action are not understood. The minimum alveolar concentrations of volatile anesthetics [the solution equivalent of 0.3 mM (Franks and Lieb, 1993)] and their rapid activity suggest an interaction with target(s) characterized as low affinity with high exchange rates. Anesthetic effects ultimately manifest as changes in protein function (Curry et al., 1990; Moss et al., 1991; Kai et al., 1998; Belelli et al., 1999), which implicates them as possible molecular targets. Investigating the role of proteins in anesthetic mechanisms is a comprehensive multidisciplinary effort, one component of which is identifying and characterizing anesthetic-protein interactions.

Anesthetics are known to bind to a variety of soluble and membrane proteins with high-micromolar to low-millimolar affinities (Curry et al., 1990; Eckenhoff and Shuman, 1993; Franks et al., 1998; Tang et al., 1999; Bhattacharya et al., 2000; Ishizawa et al., 2000). Even at these low affinities, a specific component of anesthetic binding to proteins is suggested by the absence of anesthetic binding to some proteins (Ishizawa et al., 2000; Eckenhoff et al., 2001; Xi et al., 2004) from competition studies (Moss et al., 1991), site-directed mutagenesis (Belelli et al., 1999; Carlson et al., 2000; Mascia et al., 2000; Zhou et al., 2000; Jenkins et al., 2001), and the stereospecificity of anesthetic effects (Harris et al., 1992). Based on this, techniques used to identify anesthetic-protein interactions need to be sensitive to low-affinity interactions and amenable to study proteins in aqueous or lipid environments.

The structural requirements and consequences of anesthetic binding to proteins are still unclear. The crystal structures of anesthetics bound to human serum albumin (Bhattacharya et al., 2000), firefly luciferase (Franks et al., 1998), and adenylate kinase (Sachsenheimer et al., 1977) reveal that anesthetics bind to mostly amphiphilic sites in preformed pockets and clefts, producing minimal changes to the protein structure. Some of the anesthetic binding sites spatially overlap with protein ligands or substrates, which is consistent with the (postulated) competitive inhibition of luciferase by anesthetics (Franks and Lieb, 1984; Franks et al.,

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ABBREVIATIONS: NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; STD, saturation transfer difference; τ_c, rotational correlation time; t_{sat}, saturation time, the duration of continuation rf irradiation of the sample; CaM, calmodulin; apoCaM, calcium-free CaM; holoCaM, calcium-saturated CaM; BSA, bovine serum albumin.

1998). In contrast, a molecular simulation of halothane interactions with a transmembrane channel protein in a hydrated lipid environment indicated that the rapid exchange of anesthetic molecules at protein-lipid-water interfaces altered global protein dynamics (Homanics et al., 2002; Tang and Xu, 2002). Thus, the types of anesthetic-protein interactions that can produce anesthetic effects are not clear.

Low-affinity binding interactions are difficult to characterize with most existing methods (Eckenhoff and Johansson, 1997) because of the short lifetime of the complex. Many nuclear magnetic resonance (NMR) parameters can be used for probing complex formation, which elevates high resolution NMR spectroscopy into one of the most powerful methods in drug design (Zerbe, 2003). The nuclear Overhauser effect (NOE) (Solomon, 1955; Noggle and Schirmer, 1971; Macura and Ernst, 1980), which is caused by cross-relaxation, is particularly useful for the study of weak interactions.

In double resonance NMR methods, the NOE between the irradiated (selected) spin and all its neighbors can be probed in a single experiment. In the two-dimensional NOE method (NOESY), the complete cross-relaxation network can be mapped in a single experiment (Macura and Ernst, 1980), which provides the basis for determination of macromolecular structures in solution (Wüthrich, 1986). Because of the versatility of the effect, many variants of the basic NOE experiment have been developed to probe various aspects of molecular structure and dynamics.

Saturation transfer difference (STD) is a double resonance NOE method used to probe low affinity interactions ($K_d \approx 10^{-8}$ to 10^{-3} M) between small molecules and proteins (Mayer and Meyer, 1999) and has been used for lead generation in drug discovery (Peng et al., 2001; Stockman and Dalvit, 2002). In the STD technique, saturation transfer from the protein protons to the ligand protons identifies ligand binding to a protein. The saturation transfer takes place only to molecules bound to the protein with a rate that depends on the protein mobility, ligand/protein complex lifetime, and geometry. Because in high-resolution spectroscopy the chemical shifts of small molecules are distinctive, several molecules can be screened for binding to a single protein simultaneously (Mayer and Meyer, 1999).

The goal of this study is to establish STD as a technique to screen soluble proteins for binding interactions with anesthetic molecules. The proteins bovine serum albumin (BSA), apomyoglobin, and lysozyme were screened for anesthetic binding by STD to demonstrate that results are consistent with independent techniques. STD is then used to examine the effect of protein structure on anesthetic interaction using calmodulin (CaM).

Materials and Methods

NOE Arises from Cross-Relaxation. Cross-relaxation is the incoherent magnetization exchange (Macura et al., 1981) among nuclear spins because of dipole-dipole interactions. Cross-relaxation can be investigated either in a transient mode (Solomon, 1955; Macura and Ernst, 1980; Wüthrich, 1986) or in steady-state mode (Noggle and Schirmer, 1971). Steady-state NOE (η) is the ratio between the cross-relaxation and overall relaxation rates, σ and ρ, respectively:

$$\eta = \frac{\sigma}{\rho} \tag{1}$$

and for spin pair (denoted with subscript 2):

$$\eta_2 = \frac{\sigma_2}{\rho_2}\frac{1}{1+f_{ex}} = \frac{5+\omega_0^2\tau_c^2-4\omega_0^4\tau_c^4}{10+23\omega_0^2\tau_c^2+4\omega_0^2\tau_c^2}\frac{1}{1+f_{ex}} \tag{2}$$

Parameter f_{ex} represents the relative contribution of external relaxation sources, τ_c is the correlation time of the process that modulates dipole interaction, and ω_0 is the resonance frequency of the observed nuclei. In diamagnetic systems, external relaxation is negligible, $f_{ex} \ll 1$.

In NOE Methods, Binding Is Defined by the Lifetime of the Interaction. When protein protons are irradiated, their fast cross-relaxation with other protein protons (spin diffusion) (Macura and Ernst, 1980) rapidly distributes spin saturation throughout the network of protein protons. Saturation further transfers to any bound molecule (anesthetics) that satisfies the condition that the effective correlation time (τ_{eff}), which is the combined complex lifetime (τ_B) and the protein tumbling correlation time (τ_c), is longer than the period of proton resonance frequency (ω_0):

$$\frac{1}{\tau_{eff}} = \frac{1}{\tau_B} + \frac{1}{\tau_c} < \omega_0 \tag{3}$$

This means that at resonance frequency of 500 MHz, the lifetime limit of the anesthetic-protein complex is 360 ps; shorter living complexes will not saturate by the double resonance method. Thus, the only criterion on anesthetic binding in the NOE-type experiments is an effective correlation time, i.e., average lifetime of the complex.

Cross-Relaxation from Protein to Bound Anesthetic Molecule Results in Negative NOE. The sign and the magnitude of the steady-state NOE depend on the overall mobility and structure of the complex. A model of isotropic rigid body motion (eq. 1) is adequate for screening purposes so τ_{eff} (eq. 3) is substituted for τ_c in eq. 2. This model assumes that the mobility of the free ligand is much higher than the mobility of the complex ($\tau_{free} \ll \tau_c$) and that the complex is rigid, with lifetime τ_B. In diamagnetic systems, depending on overall spin pair mobility, steady-state NOE can reach two extreme values: for small molecules where $\omega_0\tau_c \ll 1$, the NOE is positive, $\eta_2 = +1/2$, and for macromolecules $\omega_0\tau_c \gg 1$, the NOE is negative, $\eta_2 = -1$. Thus, the NOE on anesthetic molecules that bind to proteins with $\tau_B > 360$ ps will be negative because $\tau_c > 1$ ns for folded proteins.

In Multispin Systems, σ Is Larger but η Is Smaller. The NOE in a multispin system follows the same pattern as in a two-spin system, but quantitative expressions strongly depend on the selected model (Noggle and Schirmer, 1971). In general, ρ and σ are proportional to the number of participating spins (Noggle and Schirmer, 1971; Macura and Ernst, 1980); however, ρ may grow faster because equivalent spins contribute to the relaxation of each but do not contribute to σ. The overall effect is that in multispin systems, initial build-up of NOE is faster than in the spin pair, and the η is lower. For exact determination of σs, one would normally need to take into account a full network of magnetization transfer (Macura and Ernst, 1980); however, for the STD experiment, the network can be approximated by the three groups of equivalent spins: a group that is irradiated (A, one or more protein protons), a group that is not irradiated but participates in the magnetization transfer (B, protein protons), and a group at which saturation transfer is measured (C, anesthetic protons): $M_A \rightarrow M_B \rightarrow M_C$. Thus, saturation transfer from the protein to the anesthetic can be approximated by two steps: intramolecular NOE, which distributes the saturation to the anesthetic binding site ($M_A \rightarrow M_B$), and intermolecular NOE, which transfers the saturation to the bound anesthetic ($M_B \rightarrow M_C$).

Saturation Transfer Difference Spectroscopy Method. The STD NMR spectroscopy technique involves the comparison of the ^{1}H NMR spectra of a solution of protein and anesthetic(s) measured under on-resonance (I_{on}) and off-resonance (I_{off}) irradiation. In on-resonance irradiation, only the magnetization of protein protons is

affected (partially or completely saturated) but not the anesthetics or water. Off-resonance irradiation denotes that the sample is irradiated, with the same rf power and duration, far from resonance for the protein, anesthetic, or water protons. A difference spectrum (ΔI) is generated by subtracting the ^{1}H NMR spectra on-resonance from off-resonance ($\Delta I = I_{off} - I_{on}$). Nuclear magnetization of the anesthetics that do not interact with the protein is unaffected by protein pre-irradiation ($\Delta I = 0$). On the other hand, the nuclear magnetization of anesthetics that interact with the protein is partly saturated, which results in a reduction of peak integral intensity in on-resonance irradiation ($I_{off} > I_{on}$). The relative amount of saturation transferred to the anesthetic $\Delta I/I_{off}$ is the measure of the NOE on the anesthetic. With increased irradiation time (t_{sat}), $\Delta I/I_{off}$ approaches the steady-state volume, η (eq. 1).

Experimental. The STD measurements were performed on a Bruker DRX-500 MHz spectrometer employing a pulse scheme with WATERGATE water suppression and $T_{1\rho}$ filter (Mayer and Meyer, 2001). The irradiation power ($\gamma B_1/2\pi$) was 20 Hz, which was applied on-resonance at 0.4 ppm or off-resonance at 14.7 ppm. The spectra were collected in an interleaved fashion to reduce temporal fluctuations. As controls, the experiment was performed on a solution of anesthetic without protein (or protein without anesthetic) to confirm that the on- and off-resonance irradiation frequencies did not affect the anesthetic and that the protein was not saturated by the off-resonance irradiation. The relaxation delay was set to 60 s to allow the anesthetic ligands to relax between on- and off-resonance irradiation cycles. The STD spectrum collected at each t_{sat} was the sum of 16 scans. Software (XWINNMR; Bruker, Newark, DE) was used to subtract the unprocessed on- and off-resonance spectra, baseline correct the resulting difference spectrum, and integrate the areas of the anesthetic peaks.

The STD measurements were done in triplicate and representative difference spectra are presented in the figures. The intramolecular NOE in BSA was determined from the mean intensity of the BSA spectrum at 7.2, 4.0, and 1.0 ppm at each t_{sat} (measured without the $T_{1\rho}$ filter).

All experiments were performed in D_2O to eliminate influence of exchangeable protein protons. Because most of the water molecules in and around protein are highly mobile (Likic et al., 2000), water protons are poor mediators of spin diffusion; thus, water isotope exchange does not affect interpretation of STD experimental results.

Sample Preparation. Lyophilized protein was dissolved in D_2O and passed through 0.2-μm syringe filters. Protein concentrations were determined using known molar absorptivities at 280 nm. Anesthetic was added to the samples by dilution of anesthetic-saturated D_2O. The final concentrations of anesthetic in each sample was determined from the areas of the anesthetic NMR peaks relative to the external standard and are reported as the mean value of the three measurements, which had relative standard deviations of less than 20%. The buffer used for apomyoglobin was 10 mM sodium phosphate, pH 6.8. The buffer for BSA and lysozyme was 20 mM sodium phosphate, pH 7.5, 100 mM NaCl, 5 mM KCl, and 1 mM $MgSO_4$. The buffer for apoCaM was 20 mM Tris-d_{11}, pH 7.0, and 1 mM EDTA-d_{12}. The buffer for holoCaM was 20 mM Tris-d_{11}, pH 7.0, and 1 mM $CaCl_2$. NMR samples were prepared and immediately transferred to 5-mm NMR tubes. Sodium acetate in D_2O [10 mM coaxial 2-mm tube (WILMAD LABGLASS, Buena, NJ)] was used as an external standard.

Materials. D_2O was purchased from CDN Isotopes (Pointe-Claire, Quebec, Canada) and EDTA-d_{12} and Tris-d_{11} from Cambridge Isotope Laboratories (Andover, MA). Halothane [2-bromo-2-chloro-1,1,1-trifluoroethane ($CF_3CHClBr$)] was obtained from Halocarbon Laboratories (Rivers Edge, NJ), whereas sevoflurane [fluoromethyl 2,2,2,-trifluoro-1-(trifluoromethyl) ethyl ether, $CH_2FOCH(CF_3)_2$] and isoflurane (1-chloro-2,2,2-trifluoroethyl difluoromethyl ether, $CF_3CHClOCHF_2$) were purchased from Abbott Laboratories (North Chicago, IL). Fatty acid free BSA was purchased from Roche (Switzerland). Lyophilized lysozyme, horse skeletal muscle apomyoglobin, diethyl ether, and *n*-hexanol were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant CaM was expressed in *Escherichia coli* and was purified to greater than 98%, as judged by Coomassie Blue gel staining, by chromatographic separation on a phenyl-Sepharose column followed by an anion exchange column.

Results

STD Detects Halothane Binding to BSA. The ^{1}H NMR spectrum of BSA in D_2O is shown in the lower trace of Fig. 1A. The ^{1}H NMR spectrum of a solution of 1 mM halothane and external standard (sodium acetate) in D_2O is shown in the upper trace of Fig. 1A. Sodium acetate has a single ^{1}H NMR peak (three equivalent protons) at 1.9 ppm and halothane at 6.5 ppm (single proton). The off-resonance STD spectrum of BSA, and 1 mM halothane, is shown in the lower trace of Fig. 1B. The peaks of the anesthetic and external standard overlap with portions of the BSA spectrum, which is reduced by the $T_{1\rho}$ filter (upper trace of Fig. 1B).

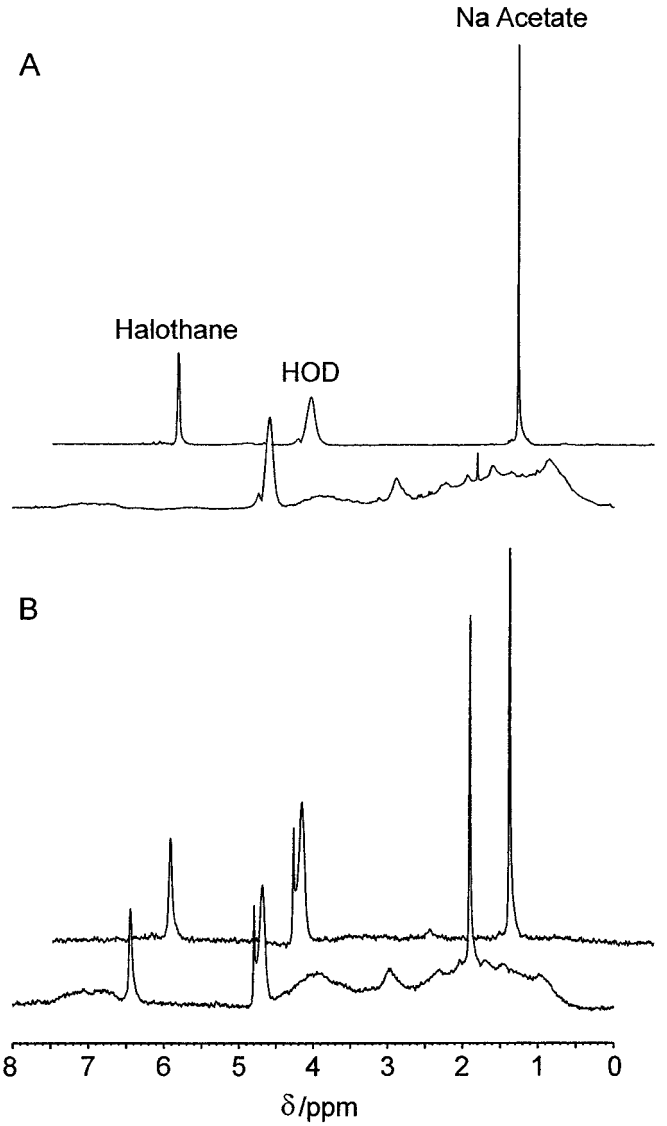

Fig. 1. A, ^{1}H NMR spectra of a solution of BSA in D_2O (lower trace) and a solution of 1 mM halothane and 10 mM sodium acetate in D_2O (upper trace, offset). B, the off-resonance STD spectra of a solution of BSA with 1 mM halothane and 10 mM sodium acetate, measured without (lower trace) and with (upper trace, offset) the $T_{1\rho}$ filter.

A representative STD experiment on BSA with 1.4 mM halothane collected at 22°C without the $T_{1\rho}$ filter is illustrated in Fig. 2. A series of ^{1}H NMR spectra collected at t_{sat} between 0.1 and 12 s with rf radiation-tuned off-resonance is shown in Fig. 2A. Spectra of the sample measured at the same power level and corresponding t_{sat}, but at an rf on-resonance for the protein but not the anesthetic (0.4 ppm), are shown in Fig. 2B. The series of difference spectra generated by subtracting B data from A are displayed in C.

STD Confirms That Anesthetics Bind to BSA. The intramolecular η in BSA was 34% at $t_{sat} \geq 2$ s (Fig. 3A). The rate of spin diffusion in BSA is much faster than the σ to halothane, which is further reduced by the addition of oleic acid (Fig. 3B). The NOE on halothane is 20 to 30% larger than the NOE on halothane measured in the presence of oleic acid and approaches the intramolecular η in BSA.

The difference spectrum of a solution of BSA with isoflurane, halothane, sevoflurane, diethyl ether, and hexanol is shown in Fig. 4. The appearance of the NMR peaks of each compound in the difference spectrum ($I_{off} > I_{on}$) indicates that each type of anesthetic molecule in the solution binds to BSA.

STD Detects Halothane Binding to Apomyoglobin but Not Lysozyme. There was an observable NOE on halothane in a solution with apomyoglobin but not lysozyme (Fig. 5).

Ca^{2+} Mediates Halothane Binding to CaM. There was a NOE on halothane in a solution with holoCaM, which was not observed with apoCaM (Fig. 6).

Discussion

The STD experiments were run under conditions that the volatile anesthetic concentration was approximately equal to the presumed dissociation constant ($K_d \approx 1$ mM) but in large excess of the protein concentration. Under these conditions, the binding site is approximately half-filled. These conditions do not preclude observing anesthetic-protein interactions with $K_d < 0.1$ mM, although such K_d values are not anticipated with volatile anesthetics.

The STD spectra indicate that halothane binds to BSA, which agrees with the findings of other methods (Dubois and Evers, 1992; Eckenhoff and Shuman, 1993). The intramolecular NOE is negative because BSA $\tau_c = 42$ ns (Visscher and Gurd, 1975; Kiihne and Bryant, 2000). The intermolecular NOE is negative, which is expected from the finding that the isoflurane-BSA complex has a lifetime of 184 μs (Dubois and Evers, 1992). The NOE buildup in halothane had a sigmoidal shape with a σ slower than the spin diffusion rate within BSA, which is consistent with the indirect multistep process of saturation transfer to halothane. The intermolecular η on halothane was similar in magnitude to the intramolecular η in BSA because the bound halothane and protein experience similar relaxation processes. Thus, the data are consistent with the theoretical description of NOE-based NMR methods and yields results that agree with other techniques.

The addition of oleic acid reduced the σ to and η in halothane, which indicates that oleic acid reduces the number of halothane binding sites in BSA. This finding is consistent with other techniques that determined approximately half of all anesthetic molecules bound to BSA occupied sites that are saturable, but displaceable with oleic acid (Eckenhoff and Shuman, 1993; Jo-

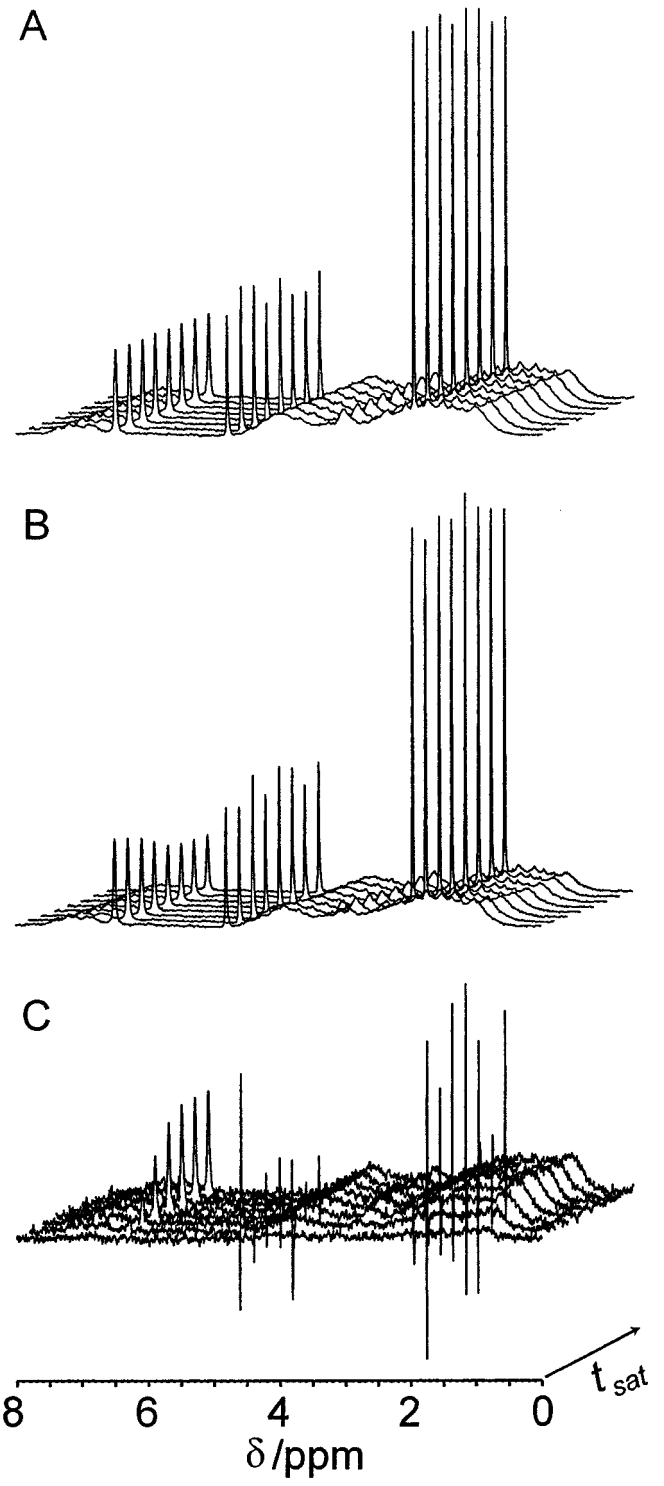

Fig. 2. Representative spectra from an STD experiment on a solution of BSA and 1.4 mM halothane collected without the $T_{1\rho}$ filter. A, spectra collected with off-resonance irradiation for various t_{sat} from 0.1 to 12 s. B, spectra collected on the same sample, but with rf radiation tuned on-resonance to BSA for the same t_{sat}. The area of the halothane and BSA but not sodium acetate NMR peaks decreases to a minimum with increasing t_{sat}. C, difference of the spectra in A from the spectra in B. The difference spectra are magnified relative to the scales in A and B for clarity. The difference spectra demonstrate the increased magnitude in the intramolecular NOE in BSA and the intermolecular NOE between BSA and halothane. The dispersive shape of both the residual water and sodium acetate peaks is caused by artifacts that arise from subtracting two narrow peaks of identical area.

hansson et al., 1995). Thus, even in a protein with a large amount of nonsaturable anesthetic binding sites, STD is sensitive to changes in anesthetic binding that result from competition with other strongly binding molecules.

STD is able to screen BSA for binding multiple anesthetics simultaneously, even if the binding sites for the different anesthetics overlap, because the high exchange rate allows different molecules access to the same site during the experiment. The anesthetics studied included an alkane (halothane), ethers (isoflurane, sevoflurane, and diethyl ether), and an alcohol (*n*-hexanol). Anesthetic binding across the range of molecules examined indicate that the anesthetic "binding site(s)" accommodates a variety of molecular structures.

STD measurements indicate that halothane binds to apomyoglobin but not lysozyme. This agrees with results obtained with other techniques (Dubois and Evers, 1992; Eckenhoff et al., 2001) and confirms that anesthetic binding sites are present in some but not all proteins (Eckenhoff et al., 2001). These results demonstrate that STD can differentiate between proteins that bind anesthetics from those that do not.

STD also found that halothane does not bind to myoglobin (data not shown). Although this result is consistent with the findings of other techniques (Eckenhoff et al., 2001), it is not a definitive negative because myoglobin contains a paramagnetic heme moiety. Fast T1 relaxation near the paramagnetic regions of proteins can quench cross-relaxation effects and prevent saturation transfer to bound anesthetic molecules. Complementary NMR methods [e.g., the influence of paramagnetic center on the free ligand relaxation time (Todorovic et al., 1999)] can be used to investigate anesthetic binding to paramagnetic protein regions. Together, the results show

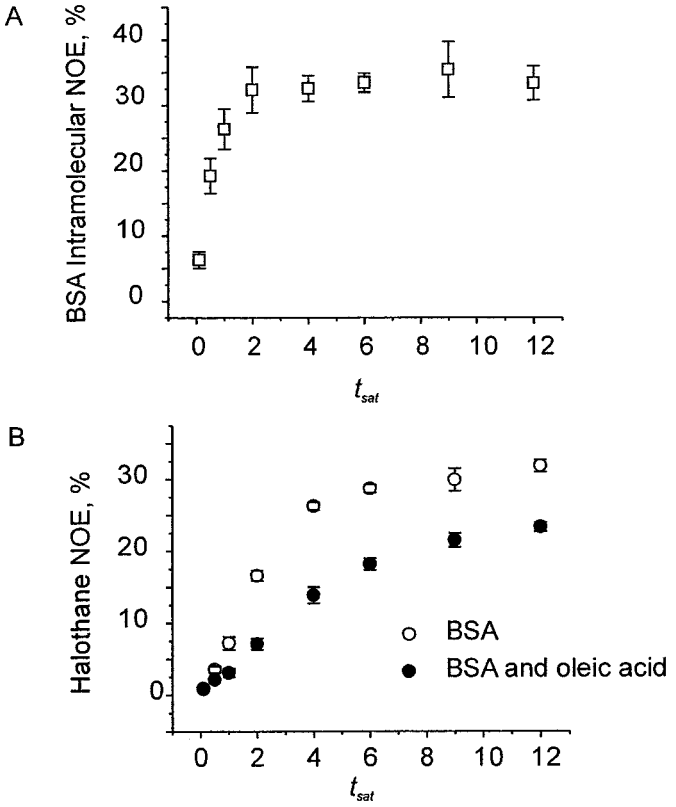

Fig. 3. A, intramolecular NOE in BSA. B, intermolecular NOE on 1.4 mM halothane from BSA (○) and on 1.6 mM halothane from BSA with 180 μM oleic acid (●).

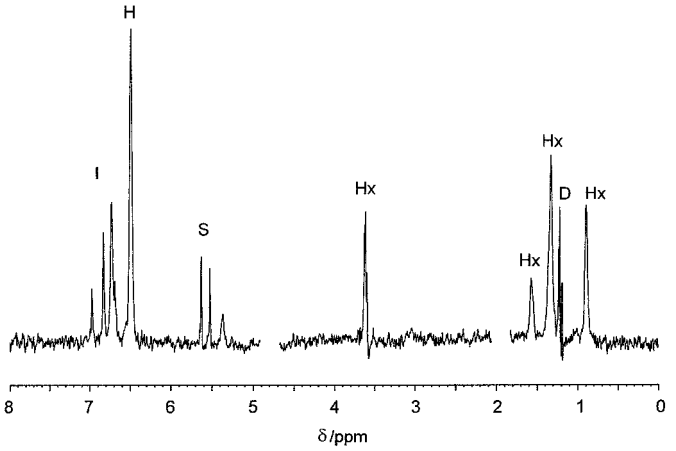

Fig. 4. Difference spectrum at t_{sat} = 12 s of a solution of BSA with isoflurane (I), halothane (H), sevoflurane (S), diethyl ether (D), and hexanol (Hx). The regions of the water and sodium acetate peaks in the spectrum are not shown.

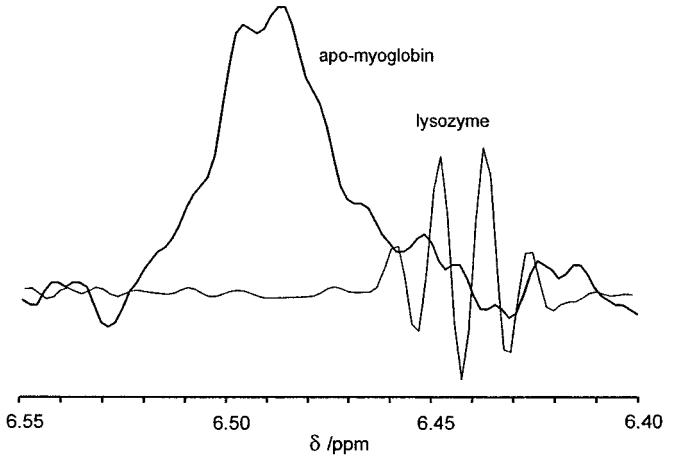

Fig. 5. Difference spectrum at t_{sat} = 15 s of solutions of apomyoglobin (thin line) or lysozyme (thick line) with halothane. The line broadening of the difference spectrum of halothane with apomyoglobin (6.42–6.46 ppm) is caused by an increase of transverse relaxation caused by binding to protein.

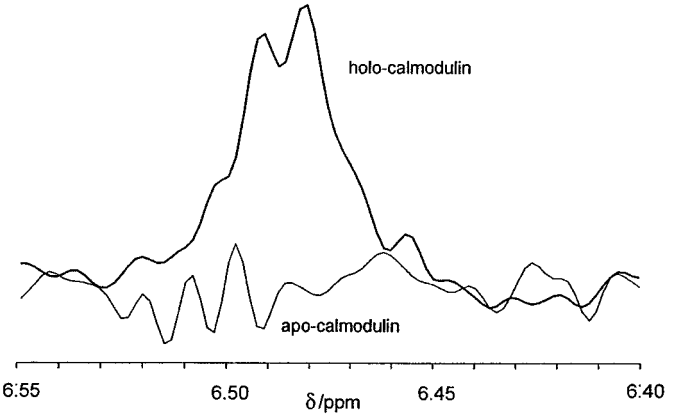

Fig. 6. Difference spectrum at t_{sat} = 15 s of solutions of apoCaM or holoCaM with halothane. The line broadening of the difference spectrum of halothane with holoCaM (6.48–6.53 ppm) is a result of increased transverse relaxation rate caused by binding.

that qualitative screening of proteins for anesthetic binding with STD in conjunction with other NMR methods, when necessary, is feasible.

STD measurements indicate that halothane binding to CaM is mediated by Ca^{2+} binding. This finding suggests that halothane might, in turn, influence Ca^{2+} binding to CaM and is supported by the report that 1 mM halothane increases the Ca^{2+} affinity of CaM (Levin and Blanck, 1995). Halothane binding to CaM is specific in that it binds selectively to the holoCaM conformation. Thus, STD is able to distinguish protein conformations that bind anesthetics from those that do not.

This suggests that halothane molecules bind to locations that are not accessible in apoCaM, such as the hydrophobic pockets on the surface of the Ca^{2+} bound EF hands (Kuboniwa et al., 1995; Zhang et al., 1995). Evidence of specific anesthetic binding sites within the hydrophobic pockets is provided by the observations that the CaM antagonist W-7 [*N*-(6-aminohexyl)-5-chloro-1-napthalene] binds in these hydrophobic pockets (Osawa et al., 1998) and compete with local anesthetics for binding to holoCaM (Tanaka and Hidaka, 1981). Because STD is related to NOESY, it is likely that these halothane-protein interactions would be observable in a structure determined with NMR. A detailed three-dimensional structure of the anesthetic holoCaM complex would help in understanding where anesthetics bind, how they alter W-7 binding, and if they affect CaM function (Tanaka and Hidaka, 1981).

The interaction of anesthetics with proteins cannot be considered universally nonspecific because they bind to some but not all proteins. It is difficult to further subdivide anesthetic-protein interactions into specific versus nonspecific because there are small differences in the affinities among the sites. Saturability of the binding site is one way to characterize the specificity of a low affinity interaction; however, with the low aqueous solubility of volatile anesthetics it may be impossible to reach saturable concentrations for many, if not most anesthetic binding sites. Finally, anesthetic-protein interactions relevant to anesthetic mechanisms may not occur at discrete saturable binding sites (Tang and Xu, 2002). Thus, although specific binding is the easiest result to interpret, it is not necessarily a prerequisite for a significant functional effect.

This is the first description of the STD technique used to screen proteins for anesthetic binding, although various other NMR methods have been used to study anesthetic-protein interactions. The interactions of anesthetics with lipids, peptides, and proteins have been studied with ^{19}F NMR (Dubois and Evers, 1992; Xu et al., 2000) and selective homonuclear NOE (Yokono et al., 1989; Tang et al., 1999) and heteronuclear NOE (Xu and Tang, 1997) methods. Selective saturation is only possible in molecules with preassigned resonances, a task that is prohibitive for protein screening and impossible for proteins much over 25 kDa. This application of STD differs from the above approaches because it uses nonselective protein saturation to answer the simple question "does the anesthetic bind to the protein?", which is fundamental to any investigation of anesthetic-protein interactions.

The STD technique could prove to be a robust method to screen for anesthetic-protein interactions. Nonexchangeable protons are abundant in proteins, ensuring that saturable protein protons will always be nearby an anesthetic binding site, regardless of the local sequence or structure. This study is confined to soluble proteins; however, STD should be able to screen for anesthetic interactions with proteins in lipid environments (see, for example, Meinecke and Meyer, 2001), which is of interest because membrane proteins are considered functionally relevant anesthetic targets.

STD is presented as a direct method for screening proteins for anesthetics binding. The nonselective character of the spin diffusion ensures that binding of anesthetic molecule at any protein surface is detected; however, any sequence-specific information about binding is lost during the long irradiation times necessary to generate η. Hence, STD cannot provide a detailed description of particular binding interactions; however, the selectivity can be regained by manipulation of the target protein (selective deuteration) or by application of a cross-relaxation editing technique (Macura et al., 1992).

STD is a powerful tool for identifying low affinity interactions between proteins and molecules such as anesthetics and alcohols. The STD technique was validated as a screening method using a variety of soluble proteins with previously determined degrees of anesthetic interaction. STD findings demonstrate that halothane binding to CaM is conformationally specific. This finding has implications on anesthetic-protein interactions and perhaps functional relevance, which is a subject of ongoing investigation. Directed screening of proteins for anesthetic binding is feasible with STD and can generate anesthetic-protein complexes for more detailed structural, dynamic, and functional studies.

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